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WITNESS my hand this
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PROVISIONAL SPECIFICATION

Invention Title: **CELL SURFACE MARKER**

Applicant: **MONASH UNIVERSITY.**

The invention is described in the following statement:

CELL SURFACE MARKER

The present invention relates to a cell surface marker, in particular to an antibody which binds to the cell surface marker and uses thereof. The invention also relates cells isolated using the antibody and uses thereof.

BACKGROUND TO THE INVENTION

Controversy exists over the nature of the stem cell or stem cells that repopulate the adult liver following various forms of injury. Interconversion between the hepatic and pancreatic endodermal lineages may result in stem cells from adult or embryonic sources offering potential for cell based transplantation therapy of liver or pancreatic diseases. However, given the uncertainty over the precise nature of the progenitor cells in the hepatic lineage, the isolation of a suitable cell type for transplantation remains problematic, as does selection and expansion of precursor cells from embryonic stem cell populations, or *ex-vivo* expansion of progenitors from foetal, neonatal or adult sources. Without the ability to identify particular hepatic endodermal stem cells, it is difficult to study these cells in isolation and to use them for the development of pharmaceuticals that might influence liver cell growth and differentiation, or the transdifferentiation of these cells into other lineages, including pancreatic.

Thus it is an object of the present invention to provide reagents and methods useful in identifying and isolating sub-populations from stem cell populations such as hepatic stem cells that may be used in repopulating liver.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a marker antibody which can bind to a marker on a sub-population of stem cells said marker further identified by a GCTM-5 antibody. Preferably, the marker antibody is a GCTM-5 antibody.

The present invention provides an antibody which can be used to identify a unique sub-population of stem cells which show characteristics of a hepatic progenitor cell. More preferably, the sub-population of stem cells is a hepatic stem cell.

The present invention also provides a hybridoma which produces a GCTM-5 antibody. Preferably, the hybridoma has a European Collection of Animal Cell Cultures accession number XXXXXX.

In another aspect of the invention, there is provided a method of identifying a sub-population of stem cells in a cell sample, said method comprising

obtaining a cell sample,

contacting the cell sample to a marker antibody which recognises a marker on the cell which is also identified by a GCTM-5 antibody and allowing the marker antibody to bind to the cell; and

identifying the cell bound to the marker antibody.

In another aspect of the invention, there is provided a method of isolating a sub population of stem cells, said method comprising

obtaining a stem cell sample,

contacting the stem cell sample to a marker antibody which recognises a marker on the cell which is also identified by GCTM-5 antibody and allowing the marker antibody to bind to the cell;

identifying the cell bound to the marker antibody; and

isolating the cell bound to the marker antibody.

In another aspect of the present invention, there is provided a sub-population of cells which express a marker which can bind to a marker antibody, wherein said marker can be further recognised by a GCTM-5 antibody.

In yet another aspect of the present invention there is provided a cell identified by a marker antibody which can bind to a marker that is further recognised by GCTM-5 antibody.

In yet another aspect of the present invention there is provided a use of the cells identified or isolated by a marker antibody which recognises a marker that is further recognised by a GCTM-5 antibody. Preferably the marker antibody is GCTM-5. The use may be selected from the group including but are not limited to, transplantation, *ex vivo* expansion, reprogramming to generate other cell types and for identifying new therapeutic agents that may affect how these cells live, grow, replicate, differentiate and die.

In a preferred aspect of the present invention there is provided a method of treating a liver disorder or other disorders that might be corrected by transplantation of GCTM-5 progenitor cells in a patient, said method comprising:

isolating a liver progenitor cell by a method including

obtaining a stem cell sample,

contacting the stem cell sample to a marker antibody which recognises a marker on a liver progenitor cell which is also identified by GCTM-5 antibody and allowing the marker antibody to bind to the cell;

identifying the liver progenitor cell bound to the marker antibody; and

isolating the liver progenitor cell bound to the marker antibody; and

transferring the liver progenitor cell into the patient.

In yet another aspect of the present invention there is provided a stem cell marker identified by GCTM-5 antibody. Preferably the stem cell marker is an endodermal or hepatic stem cell marker.

The present invention further provides a method of diagnosing or monitoring a liver disorder in a patient, said method comprising detecting GCTM-5 antigen in a biological sample. Preferably the biological sample is a body fluid sample.

The present invention also provides compositions including molecules of the present invention, and methods for their use. Preferably, the molecules are selected from the group including: a marker antibody which recognises a marker on

a cell which is also identified by a GCTM-5 antibody, or fragments or derivatives thereof; a marker on the cell which is identified by a GCTM-5 antibody; agonists of the marker and antagonists of the marker.

In yet another aspect of the present invention, there is provided an agonist which enhances a biological function of a marker on a cell, wherein the marker is identified by a GCTM-5 antibody. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided an antagonist which inhibits a biological function of a marker on a cell, wherein the marker is identified by a GCTM-5 antibody. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided a composition including a marker antibody which recognises a marker on a sub-population of stem cells, wherein the marker is also identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the stem cell is a hepatic stem cell or a pancreatic stem cell. Preferably the marker antibody is a GCTM-5 antibody.

In yet another aspect of the present invention, there is provided a composition including a marker, wherein the marker is identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the marker is a GCTM-5 antigen. Preferably the marker identifies a sub-population of stem cells. Preferably the stem cell is a hepatic stem cell or a pancreatic stem cell.

In yet another aspect of the present invention, there is provided a composition including an agonist of a marker, wherein the marker is identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided a composition including an antagonist of a marker, wherein the marker is identified by a GCTM-5

antibody, and a pharmaceutically acceptable carrier. Preferably the marker is a GCTM-5 antigen.

In particular embodiments, compositions of the present invention may be used to treat a subject. A subject in need of treatment may have a liver disorder or a pancreatic disorder. The provision of antibodies, agonists or antagonists may provide a beneficial outcome to the subject.

In yet another aspect of the present invention, there is provided a method of treating a subject including the step of administering to the subject, an effective amount of a composition, wherein the composition includes a marker antibody which recognises a marker on the cell which is also identified by a GCTM-5 antibody, and wherein the subject has a disorder selected from the group including a liver disorder and a pancreas disorder. Preferably the antibody is a GCTM-5 antibody.

In yet another aspect of the present invention, there is provided a method of treating a subject including the step of administering to the subject, an effective amount of a composition, wherein the composition includes an agonist of a marker on the cell, wherein the marker is also identified by a GCTM-5 antibody, and wherein the subject has a disorder selected from the group including a liver disorder and a pancreas disorder. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided a method of treating a subject including the step of administering to the subject, an effective amount of a composition, wherein the composition includes an antagonist of a marker on the cell, wherein the marker is also identified by a GCTM-5 antibody, and wherein the subject has a disorder selected from the group including a liver disorder and a pancreas disorder. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided a kit for detecting a marker, wherein the kit includes a marker antibody which recognises the marker and wherein the marker is also identified by a GCTM-5 antibody. Preferably the

marker antibody is a GCTM-5 antibody. Preferably the kit may be used to detect the marker in a biological sample. Preferably the biological sample is a body fluid or a tissue sample.

In yet a further aspect of the present invention, there is provided a kit for detecting a marker on a sub-population of stem cells, wherein the kit includes a marker antibody which recognises the marker and wherein the marker is also identified by a GCTM-5 antibody. Preferably the stem cell is a hepatic stem cell or a pancreatic stem cell. Preferably the antibody is a GCTM-5 antibody.

DESCRIPTION OF THE FIGURES

Figure 1 shows immunostaining revealing GCTM-5 expression in GCT27X-1 embryonal carcinoma cells. (A) shows a brightfield image of GCT27X-1 morphology and density, and (B) shows the rare expression of GCTM-5. Magnification 400X

Figure 2 shows a Western blot of GCTM-5 antigen from differentiated human ES cell cultures.

Figure 3 shows an immunohistochemical analysis of GCTM-5 expression on live novel endodermal cell line; GCTM-5 staining (pink) a subpopulation of these cells. Magnification of 100X.

Figure 4 shows an immunohistochemical analysis of 7 week human fetal tissues revealing GCTM-5 expression exclusively in the liver (A), whilst negative in fetal (B) gut (seen here juxtaposed with positive liver), (C) genital ridge, (D) lung, (E) heart and (F) notochord (arrow).

Figure 5 shows dual fluorescent immunostaining with GCTM-5 (Texas Red) and CK-19 (Fluorescein isothiocyanate) on normal and diseased human liver tissue sections. Bile ducts co-expressed both markers appearing yellow (arrow) in normal (A) and PBC liver (B). The arrowhead shows some CK-19 positive ductular reactive cells in PBC (B). In diseased tissue were ductular reactive cells were present there

was some co-localisation of GCTM-5 and CK-19, however cells close to hepatocyte margins were CK-19 positive only (Green, arrowhead). Original magnification x200.

Figure 6 shows immunocharacterization of GCTM-5 (A,C,E,G) or HEA (B,D,F,H) immunoisolated cells from PBC or ALD liver after 6 days in culture. The GCTM-5 and HEA-isolated cells were remarkably similar to each other expressing BEC phenotype (CK-19 and HEA positive; A-D). Both sets of cells were positive for GCTM-5 (E,F) and negative for the endothelial cell marker, CD31 (G,H). Cells were isolated from PBC (A,B,E-H) and ALD (C,D). Original magnification x200.

DETAILED DESCRIPTION

In a first aspect, the present invention provides a marker antibody which can bind to a marker on a sub-population of stem cells said marker further identified by a GCTM-5 antibody. Preferably, the marker antibody is a GCTM-5 antibody.

The present invention provides an antibody which can be used to identify a unique sub-population of stem cells which show characteristics of a hepatic progenitor cell. More preferably, the sub-population of stem cells is a hepatic stem cell. Even more preferably, the cells are proliferating hepatic progenitor cells.

The term "hepatic stem cell" may be used interchangeably with "liver stem cell" and encompasses within its scope a hepatoblast, an embryonic liver foetal cell, liver or hepatic progenitor cells or biliary cells, preferably biliary epithelial cells. Most preferably these cells have the capacity to proliferate in the liver. The hepatoblast is a multi-potential cell which has the capacity to differentiate to hepatocytes, biliary cells or pancreatic cells. It is now found that the cells identified by the antibody, preferably GCTM-5 antibody, can identify this cell type which has the propensity to differentiate into liver, hepatic or pancreatic cells. The possible interconversion between liver and pancreatic cells shows that the antibody is capable of being used to identify potential pancreatic cells as well as liver cells.

Throughout the description and claims of this specification the word "comprise", and variations of the word such as "comprising" and "comprises", is not intended to exclude other additives or components or integers or steps.

The antibody of the present invention may be a monoclonal or polyclonal antibody or a recombinant antibody. Preferably, the antibody is any antibody specific for a marker identified by a GCTM-5 antibody or a fragment thereof. The antibody of the present invention encompasses any antibody or fragment thereof, either native or recombinant, synthetic or naturally-derived, monoclonal or polyclonal which retains sufficient specificity to bind specifically to the marker or a fragment thereof which is indicative of an antigen identified by GCTM-5 antibody. As used herein, the term "antibody" or "antibodies" include the entire antibody and antibody fragments containing functional portions thereof. The term "antibody" includes any monospecific or bispecific compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region to effect binding to the epitope to which the whole antibody has binding specificity. The fragments can include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and include, but are not limited to, Fab fragments, $F(ab')_2$ fragments, and Fv fragments.

The recombinant antibody can be produced by any recombinant means known in the art. Such recombinant antibodies include, but are not limited to, fragments produced in bacteria and non-human antibodies in which the majority of the constant regions have been replaced by human antibody constant regions. In addition, such "humanized" antibodies can be obtained by host vertebrates genetically engineered to express the recombinant antibody.

The antibodies or fragments thereof may be obtained by methods known in the art for production of antibodies or functional portions thereof. Such methods include, but are not limited to, separating B cells with cell-surface antibodies of the desired specificity, cloning the DNA expressing the variable regions of the light and heavy chains and expressing the recombinant genes in a suitable host cell. Standard

monoclonal antibody generation techniques can be used wherein the antibodies are obtained from immortalized antibody-producing hybridoma cells. These hybridomas can be produced by immunizing animals with HSCs or progeny thereof, and fusing B lymphocytes from the immunized animals, preferably isolated from the immunized host spleen, with compatible immortalized cells, preferably a B cell myeloma.

The antibodies or fragments thereof may be obtained from any source. Effectively, any means which detects the presence of a marker identified by GCTM-5 antibody or fragments of the marker on the cells is within the scope of the present invention.

The marker is any molecule or antigen on the sub-population of stem cells which is indicative of the sub-population of stem cells. Preferably, it is indicative of a hepatic stem cell. The molecule indicative of the marker may be an epitope or a portion of an epitope that can be identified by the antibody GCTM-5 or a fragment of the antibody that is functionally the same as the whole GCTM-5 antibody. The marker may also be identified by a marker antibody which may be a polyclonal antibody which also recognises the epitope defined by the GCTM-5 antibody or a functionally active fragment thereof.

The marker antibody and the GCTM-5 antibody can recognise the same epitope. The marker antibody can be tested for its suitability by its ability to compete against the GCTM-5 antibody.

The preferred antibody is GCTM-5 antibody which is a monoclonal antibody which was raised against a membrane preparation from a testicular seminoma tumour. Surprisingly, screening of this reagent against a pluripotent human embryonal carcinoma cell line revealed that it bound to a minority cell population in the culture that was unreactive with markers of pluripotent stem cells. GCTM-5 antigen is now found to be expressed exclusively in the foetal liver of a seven week human foetus. Concurrent experimentation on normal and diseased human liver tissue reveal an expression localised to the ductal region. Hence the applicants have found a

means to identify a sub-population of cells based on expression of a GCTM-5 antigen or equivalent which is identified by the antibody GCTM-5.

The present invention also provides a hybridoma which produces a GCTM-5 antibody. Preferably, the hybridoma has a European Collection of Animal Cell Cultures accession number XXXXXX.

The "equivalent" as used herein with respect to the antigen or marker is a molecule that can elicit a binding or recognition of the antibody GCTM-5. Therefore, it too can be identified by GCTM-5 antibody but may not be the exact epitope of GCTM-5. The equivalent may have mutations that do not substantially affect the binding or recognition by GCTM-5. Due to the stringency of the binding, equivalents can be identified which are partially identified by GCTM-5.

In another aspect of the invention, there is provided a method of identifying a sub-population of stem cells in a cell sample, said method comprising

obtaining a cell sample,

contacting the cell sample to a marker antibody which recognises a marker on the cell which is also identified by a GCTM-5 antibody and allowing the marker antibody to bind to the cell; and

identifying the cell bound to the marker antibody.

The present invention has identified a marker which is indicative of a unique sub-population of stem cells. Preferably the sub population of stem cells are hepatic stem cells. The sub-population of hepatic stem cells includes hepatoblasts, embryonic liver foetal cells, liver or hepatic progenitor cells, biliary cells, biliary epithelial cells, multi-potential cells which can differentiate to hepatocytes, biliary cells and pancreatic cells and cells of the luminal surfaces of biliary ducts, or other cell types of endodermal origin. Preferably the hepatic stem cells are hepatic and liver progenitor cells which have the capacity to proliferate. Therefore, the method is useful for identifying hepatic progenitor cells rapidly proliferating and differentiating in liver cancers.

The cell sample may be from any source of biological material, but is not limited to, blood, tissues, sputum, urine, and faecal samples. The cells may also be cell cultures which include progenitor cells such as, but not limited to, an ES cell culture or HES cell culture of undifferentiated cells or pluripotent stem cells or any hepatic cell culture which includes adult and foetal or undifferentiated liver or hepatic cells. The cell sample may be a diseased liver sample including hepatic stem cells. The cell sample may be any sample in which a cell surface is exposed for identification by the antibody. The marker that is indicative of the sub-population is a cell surface marker.

The marker antibody is as described above for the antibody in the first aspect. Preferably the antibody is GCTM-5. However, it should be appreciated that whilst the GCTM-5 antibody can recognise the marker that identifies the hepatic stem cells, any antibody which can recognise the same epitope or an equivalent or a portion thereof can be used as a marker antibody.

The cell sample is contacted to the marker antibody by exposing the sample to the marker antibody. Methods known to the skilled addressee for immunohistochemistry using an antibody which recognises an antigen which is also identified by GCTM-5 antibody may be employed. The use of primary and secondary antibodies to further enhance the identification can be employed.

To assist in identification of the cells bound to the marker antibody, the antigen can be conjugated to other suitable molecules and compounds including, but not limited to, enzymes, magnetic beads, colloidal magnetic beads, haptens, fluorochromes, metal compounds, radioactive compounds or drugs. The enzymes that can be conjugated to the antibodies include, but are not limited to, alkaline phosphatase, peroxidase, urease and β-galactosidase. The fluorochromes that can be conjugated to the antibodies include, but are not limited to, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, phycoerythrin, allophycocyanins and Texas Red. The metal compounds that can be conjugated to the antibodies include, but

are not limited to, ferritin, colloidal gold, and particularly, colloidal superparamagnetic beads. The haptens that can be conjugated to the antibodies include, but are not limited to, biotin, digoxigenin, oxazalone, and nitrophenol. The radioactive compounds that can be conjugated or incorporated into the antibodies are known to the art, and include but are not limited to technetium 99m, ^{125} I and amino acids comprising any radionuclides, including, but not limited to ^{14} C, ^{3} H and ^{35} S.

The sample may be stained to further identify the hepatic stem cells as listed above.

The marker antibody, preferably the GCTM-5 antibody therefore provides a reagent with particularly valuable properties and applications such as the identification of specific populations of cells, preferably, liver stem cells. Given the expression pattern of the GCTM-5 antigen, such populations of cells may include particular populations of stem cells such as liver or hepatic stem cells or bipotential liver/pancreatic stem cells which may also differentiate to biliary cells, preferably biliary epithelial cells, or other endodermal progenitor cells.

In another aspect of the invention, there is provided a method of isolating a sub population of stem cells, said method comprising

obtaining a stem cell sample,

contacting the stem cell sample to a marker antibody which recognises a marker on the cell which is also identified by GCTM-5 antibody and allowing the marker antibody to bind to the cell;

identifying the cell bound to the marker antibody; and

isolating the cell bound to the marker antibody.

By using the unique marker identified by the GCTM-5 antibody, the sub-population of cells that carry this marker can be isolated from the cell sample. The sample may be as described above, but it is preferred that the cell sample is a cell suspension which can facilitate the isolation and identification of the cells. However a cell culture on culture plates may be equally used and the identified cells isolated or "plucked" form the culture plate surface possibly for further culturing and

expansion. Alternatively, cell suspensions or suspensions of aggregates of cells may be isolated from tissues including liver by methods such as those used in Example 4. The subpopulation of cells within a tissue that can react with the marker may be isolated by flourescence activated cell sorting, binding to superparamagnetic beads, panning etc.

Methods are available to the skilled addressee for isolating cells from a cell sample which utilise an antibody. However, the present method utilises a unique marker identified by a GCTM-5 antibody or another antibody that recognises the same marker as GCTM-5 and a means to identify and isolate a sub-population from a stem cell population which have the propensity to differentiate to liver or pancreatic cells. Preferably the cell is a liver progenitor cell.

As described above, the antibody can be conjugated to suitable molecules, compounds and supports to facilitate the isolation of the cells by methods available to the skilled addressee. Some of the methods of isolating cells using antibodies include, but are not limited to:

capture methods where antibody bound to the surface of a cell allows the cell to be physically bound to a surface and thereby separated from other cells; and

sorting methods, such as magnetic separation and fluorescence activated cell sorting, where a magnetic or fluorescent reagent (respectfully) is bound to the antibody bound to the cell surface and separation is based on the magnetic or fluorescent properties of the cell.

GCTM-5 antibody is reactive with a cell surface epitope, which provides the potential for this reagent to be used to isolate live cellular populations, by immunomagnetic separation, or by flow cytometry. Exploitation of stem cell populations, both adult and ES cell derived, for cell-based therapies for is dependent upon isolation and characterisation of early precursor populations. GCTM-5 may be a useful tool for the isolation of liver stem cells from diseased adult

or pediatric liver, normal foetal or embyonic liver, or normal pediatric or adult liver tissue.

In another aspect of the present invention, there is provided a sub-population of cells which express a marker which can bind to a marker antibody, wherein said marker van be further recognised by a GCTM-5 antibody.

The present invention identifies a unique sub-population of cells. Preferably it is a sub-population of stem cells. The cells that comprise the sub population may be selected from the group including hepatoblasts, embryonic liver foetal cells, liver or hepatic progenitor cells, biliary cells, biliary epithelial cells, multi-potential cells which can differentiate to hepatocytes, biliary cells and pancreatic cells and cells of the luminal surfaces of biliary ducts. Preferably the hepatic stem cells are hepatic and liver progenitor cells that have the capacity to proliferate. The sub population is not necessarily a pure population of any particular cell type since any one of the above listed cells can express the marker. The sub-population may also represent cells of various degrees of differentiation. For instance, the cells may be multi-potential cells that can differentiate to hepatocytes, pancreatic cells or biliary cells or other endodermal cells. Accordingly, the sub population may comprise a mixture of these cells.

In yet another aspect of the present invention there is provided a cell identified by a marker antibody which can bind to a marker that is further recognised by GCTM-5 antibody.

The marker antibody and the GCTM-5 antibody can recognise the same epitope. Hence the marker can be identified by competition studies based on the marker antibody and GCTM-5 antibody. Similarly, the marker antibody can be tested for its suitability by its ability to compete against he GCTM-5 antibody. The cell may be selected from the group including hepatoblasts, embryonic liver foetal cells, liver or hepatic progenitor cells, biliary cells, biliary epithelial cells, multi-potential cells which can differentiate to hepatocytes, biliary cells and pancreatic cells and cells of

the luminal surfaces of biliary ducts. Preferably the hepatic stem cells are hepatic and liver progenitor cells that have the capacity to proliferate. Preferably the cell is a hepatic stem cell. More preferably, the cell is a hepatic progenitor cell. Most preferably the cell is a proliferating hepatic progenitor cell.

In yet another aspect of the present invention there is provided a use of the cells identified or isolated by a marker antibody which recognises a marker that is further recognised by a GCTM-5 antibody. Preferably the marker antibody is GCTM-5. The use may be selected from the group including but not limited to, transplantation, *ex vivo* expansion, reprogramming to generate other cell types and for identifying new therapeutic agents that may affect how these cells live, grow, replicate, differentiate and die.

In a preferred aspect of the present invention there is provided a method of treating a liver disorder in a patient, said method comprising:

isolating a liver progenitor cell by a method including

obtaining a stem cell sample,

contacting the stem cell sample to a marker antibody which recognises a marker on a liver progenitor cell which is also identified by GCTM-5 antibody and allowing the marker antibody to bind to the cell;

identifying the liver progenitor cell bound to the marker antibody; and

isolating the liver progenitor cell bound to the marker antibody; and

transferring the liver progenitor cell into the patient.

Under normal circumstances, hepatocytes are almost uniformly quiescent. However, on demand, resting hepatocytes are able re-enter the cycle and proliferate for up to 12 doublings per cell. In addition to the regenerative capacity of hepatocytes, the liver possesses a stem cell population that is multipotent and is activated by massive liver necrosis or cirrhosis. These liver stem cells reside in the terminal bile ductules. Oval cells are thought to be the daughter cells of these true stem cells, and also reside in the terminal bile ductule. Oval cells have been shown to differentiate to hepatocyte and bile ductular cells to repopulate diseased liver.

The ductal regions are the site of proliferation of these stem cell populations, as they spread and integrate into the surrounding mesenchyma.

Therefore, liver cells can be repopulated by the use of a progenitor cell capable of differentiating to the liver cell.

Liver disorders in the present application are generally those disorders that require replenishment or regeneration of liver cells. Such conditions occur in diseases such as primary biliary cirrhosis (PBC), extrahepatic biliary atresia (EHBA) or alcoholic liver disease (ALD).

Preferably cells isolated using any of the methods described above could be directly used in transplantation therapy of patients with liver disease, or they could be subject to expansion *ex vivo*. Alternately, the marker antibody or the GCTM-5 antibody may be used to isolate progenitors from other embryonic foetal pediatric or adult tissues, or to isolate progenitors from cultures of embryonic stem cells, embryonic germ cells, adult stem cells, or stem or progenitor cells derived by reprogramming of gene expression. In addition to potential uses in transplantation, the marker antibody or the GCTM-5(+) cells or their progeny may be studied *in vitro* to identify new targets for protein, nucleic acid, or small molecule therapeutics.

In yet another aspect of the present invention there is provided a stem cell marker identified by GCTM-5 antibody. Preferably the stem cell marker is a hepatic or endodermal stem cell marker. The stem cell marker of the present invention identifies a unique sub-population of stem cells that preferably show characteristics of hepatic stem cells or hepatic progenitor cells. The marker is an antigen of GCTM-5 and may be an endodermal cell marker, and more specifically an early liver marker, which could prove a useful tool for the isolation of liver progenitors for both diseased adult liver and differentiating human embryonic stem cells. More preferably, the marker is a cell surface marker.

The cell surface marker recognized by the GCTM-5 antibody may be isolated using affinity techniques or other means of protein purification or by expression cloning or other techniques known by those skilled in the art.

The cell surface marker or the antigen identified by the GCTM-5 antibody may be a polypeptide which migrates in an SDS-PAGE gel with an apparent molecular weight of 50kDa.

The present invention further provides a method of diagnosing or monitoring a liver disorder in a patient, said method comprising detecting GCTM-5 antigen in a biological sample. Preferably the biological sample is a body fluid sample.

It is envisaged that the GCTM-5 antigen may be shed or secreted from the cell surface. Accordingly, the levels of the GCTM-5 antigen may indicate the level of liver progenitor activity. Accordingly, the levels of the antigen in blood or other body fluids, which could be measured by radioimmunoassay, ELISA or other assays based on GCTM-5 or derivative reagents, could be used to diagnose or to monitor progression and treatment of hepatic and other disorders.

As a marker of a stem cell, the GCTM-5 antigen provides a useful target for identification and isolation of stem cells. The antigen may also be used as a target of protein, nucleic acid or small molecule therapeutic agents for treatment of stem cells.

The antigen reactive with GCTM-5 antibody may be isolated using affinity techniques or other means of protein purification or by expression cloning. The antigen may be used as a target of protein, nucleic acid or small molecule therapeutic agents. GCTM-5(+) precursors or their progeny may also be capable of differentiation into pancreatic or other lineages and may be useful in treatment of other conditions by cell therapy, or they may be used in laboratory research to develop new pharmaceuticals for treatment of a range of disorders.

The present invention also provides compositions including molecules of the present invention, and methods for their use. Preferably, the molecules are selected from the group including: a marker antibody which recognises a marker on a cell which is also identified by a GCTM-5 antibody, or fragments or derivatives thereof; a marker on the cell which is identified by a GCTM-5 antibody; agonists of the marker and antagonists of the marker.

In yet another aspect of the present invention, there is provided an agonist which enhances a biological function of a marker on a cell, wherein the marker is identified by a GCTM-5 antibody. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided an antagonist which inhibits a biological function of a marker on a cell, wherein the marker is identified by a GCTM-5 antibody. Preferably the marker is a GCTM-5 antigen.

Agonists and antagonists of the marker may provide useful molecules to modulate the function of the marker. The agonists and antagonists may also modulate the function of a cell which expresses the marker on its surface. Such agonists and antagonists may be identified by any method known to those skilled in the art. The method may include, but not be limited to, a competition assay wherein binding to the marker of the agonist or antagonist may inhibit binding of a GCTM-5 antibody.

In yet another aspect of the present invention, there is provided a composition including a marker antibody which recognises a marker on a sub-population of stem cells, wherein the marker is also identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the stem cell is a hepatic stem cell or a pancreatic stem cell. Preferably the marker antibody is a GCTM-5 antibody.

In yet another aspect of the present invention, there is provided a composition including a marker, wherein the marker is identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the marker is a GCTM-5 antigen.

Preferably the marker identifies a sub-population of stem cells. Preferably the stem cell is a hepatic stem cell or a pancreatic stem cell.

In yet another aspect of the present invention, there is provided a composition including an agonist of a marker, wherein the marker is identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided a composition including an antagonist of a marker, wherein the marker is identified by a GCTM-5 antibody, and a pharmaceutically acceptable carrier. Preferably the marker is a GCTM-5 antigen.

The composition may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain additional agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the antibody may be in powder form for reconstitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

In particular embodiments, compositions of the present invention may be used to treat a subject. A subject in need of treatment may have a liver disorder or a pancreatic disorder. The provision of antibodies, agonists or antagonists may provide a beneficial outcome to the subject.

In yet another aspect of the present invention, there is provided a method of treating a subject including the step of administering to the subject, an effective amount of a composition, wherein the composition includes a marker antibody which recognises a marker on the cell which is also identified by a GCTM-5 antibody, and wherein the

subject has a disorder selected from the group including a liver disorder and a pancreas disorder. Preferably the antibody is a GCTM-5 antibody.

In yet another aspect of the present invention, there is provided a method of treating a subject including the step of administering to the subject, an effective amount of a composition, wherein the composition includes an agonist of a marker on the cell, wherein the marker is also identified by a GCTM-5 antibody, and wherein the subject has a disorder selected from the group including a liver disorder and a pancreas disorder. Preferably the marker is a GCTM-5 antigen.

In yet another aspect of the present invention, there is provided a method of treating a subject including the step of administering to the subject, an effective amount of a composition, wherein the composition includes an antagonist of a marker on the cell, wherein the marker is also identified by a GCTM-5 antibody, and wherein the subject has a disorder selected from the group including a liver disorder and a pancreas disorder. Preferably the marker is a GCTM-5 antigen.

The term "effective amount" means a dosage sufficient to provide treatment or prevention for the disease or condition being treated or prevented. This will vary depending on the subject and the disease/condition being effected. The effective amounts of an agent used in the methods of the present invention may vary depending upon the manner of administration, the condition of the subject to be treated, and ultimately will be decided by the attending scientist, physician or veterinarian.

In yet another aspect of the present invention, there is provided a kit for detecting a marker, wherein the kit includes a marker antibody which recognises the marker and wherein the marker is also identified by a GCTM-5 antibody. Preferably the marker antibody is a GCTM-5 antibody. Preferably the kit may be used to detect the marker in a biological sample. Preferably the biological sample is a body fluid or a tissue sample.

As used herein, the term "biological sample" is intended to encompass cellular and non-cellular biological material, including, but not limited to, cell cultures, tissue cultures, conditioned medium, tissue samples, blood, serum, other bodily fluids and biopsy samples.

In yet a further aspect of the present invention, there is provided a kit for detecting a marker on a sub-population of stem cells, wherein the kit includes a marker antibody which recognises the marker and wherein the marker is also identified by a GCTM-5 antibody. Preferably the stem cell is a hepatic stem cell or a pancreatic stem cell. Preferably the antibody is a GCTM-5 antibody.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of this application.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

EXAMPLES

Example 1: Derivation and Characterisation of Antibodies from Hybridoma Culture.

(a) Derivation of GCTM-5 Antibody

Female BalbC mice of 4-6 weeks of age were immunised with a crude membrane preparation from a testicular seminoma. Fresh tumour tissue was disrupted by Dounce homogenisation, and the homogenate was first subjected to low speed centrifugation at 3,000xg to remove nuclei, mitochondria, cytoskeletal elements and debris, then high speed centrifugation at 100000xg for one hour. The resulting

pellet was used for immunisation. Fusion with myeloma cell line NS-1 and subsequent selection of hybridomas was carried out using standard techniques. Hybridoma supernatants were first screened by antibody capture ELISA using the immunising membrane preparation. Secondary screening was performed using either immunocytochemistry on fixed or frozen sections of seminomas, or by indirect immunofluorescence on fixed slides of cultured embryonal carcinoma cell lines including GCT27X-1 (Figure 1).

(b) Antibody Isotyping

GCTM-5 antibody was isotyped using the ISOStrip Kit (Roche Diagnostics, USA) according to manufacturer's instructions. Briefly, the hybridoma supernatant was diluted 1:20 in calcium/magnesium-free phosphate buffered saline (PBS-, Gibco BRL, Melbourne), and 150µl of this was added to the ISOStrip tube containing latex beads. This was incubated at room temperature (RT) for 30 seconds, before vortexing to resuspend the latex beads. The ISOStrip strip was placed in the tube and incubated at RT for 5-10 minutes. The latex beads migrate at a controlled rate, relative to the isotype of the antibody in the supernatant which can be read off the strip using the isotype markers.

(c) Summary of GCTM-5 expression in cultured cell lines and isolated cells

Immunofluorescent analysis identified a number of cell lines which were positive or negative for GCTM-5 expression. Reactivity of the antibody was first noted with a small subpopulation of differentiated cells in cultures of human embryonal carcinoma cell line GCT27X-1. Table 1 shows a summary of the reactivity of the antibody with cultured cell lines, including adult hepatocarcinoma cell line (HepG2), human embryonal kidney (HEK293), and EC cell lines GCT27X-1, GCT72 and GCT44.

Table 1: GCTM-5 expression analysis in a variety of cultured cell lines and isolated cell types.

Cell Lines	GCTM-5 Expression
HepG2	-
HEK293-T	-
GCT27X-1	+
GCT44	-
GCT72	+
HES (undiff)	+
Isolated Cells*	
Mononuclear Cells from human Umbilical Cord Blood	-
Biliary Epithelial Cells from PBC Liver	+++

GCTM-5 was noted as either (-) = negative expression, (+) = rare expression, and (++) = strong expression.

Example 2: Characterisation of Antigen Recognised By GCTM-5 Antibody.

(a) Western Blot Analysis of GCTM-5 Antigen:

GCT27X-1 cells were grown in 12-well plates. Cells in 4 wells of a plate was lysed with Laemmli sample buffer containing 0.2M dithiothreitol (DTT). The sample was run 10% SDS polyacrylamide gel was with a BENCHMARK Prestained Protein Ladder (Invitrogen, Victoria). The protein was then transferred to Hybond-P membrane (Amersham Biosciences, Australia) and membrane blotted with GCTM-5 (neat), or CAM5.2 (BD Biosciences, Australia) as a positive control for the cell preparation. An anti-mouse Ig conjugated to horse-radish peroxidase (HRP) secondary antibody (Dako, NSW) was added at a dilution of 1:10,000 in TBS-Tween20. Chemiluminescent ECL Reagent (Amersham Biosciences, Australia) was added to the membrane for 5 minutes, before exposure of the membrane to Hyperfilm (Amersham Biosciences, Australia).

(b) Immunohistochemistry

Double immunostaining studies of antigen co-localisation in normal and diseased adult liver tissue was performed using the fluorescent conjugates, Texas Red (IgG1) or Fluorescein isothyocyanate (FITC IgG2a, Cambridge BioScience, Cambridge UK) on 5 μm cryostat sections fixed in acetone as described previously. The primary antibody combination consisted of CK-19 (IgG2a, 1:10, Progen, Heidelberg, Germany) with GCTM-5 (IgG1, neat supernatant).

Human cell lines grown on 12-well slides were fixed with 100% ethanol. For indirect immunofluorescent analysis, a combination of primary antibody GCTM-5 (IgG1, neat supernatant) and fluorescein-conjugated secondary antibody, anti-mouse Ig-FITC (1:40, Dako, Australia) was used. Cell nuclei were then stained with 1μg/ml 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Sigma, NSW).

(c) Identification of Cell Surface Localisation of GCTM-5 Antigen:

Live immuno-staining involved the addition of the primary antibody to live cells, prior to fixation. The live cells were incubated with GCTM-5 antibody for 10 minutes at 4°C. The cells were gently washed with PBS 3X and fixed in cold 100% Ethanol for 5 minutes. The AP-conjugated secondary antibody was applied and the staining proceeded as described *supra.*

(d) GCTM-5 antigen characterisation

Western blot analysis on a whole cell lysate of putative endodermal cells revealed a GCTM-5 protein band of approximately 50kDa (Figure 2). Lanes probed with the secondary antibody only showed no staining of the 50kDa band.

(e) Cell surface localisation of GCTM-5

The staining pattern observed with GCTM-5 on GCT27X-1 cells was consistent with surface or pericellular staining. Incubation of live cells at 4°C with the antibody prior to fixation revealed that the epitope reactive with the reagent was indeed localised to the outside of the cell (Figure 3).

Example 3 - Staining of Human Foetal Sections

(a) Foetal Tissue

Histological sections of first trimester human embryos were cut from archival material obtained with informed consent from patients undergoing termination of pregnancy at the John Radcliffe Hospital, Oxford, UK. The tissue was fixed in absolute alcohol, embedded in paraffin, and sectioned at 5μM thickness.

Paraffin embedded human foetal sections for immunohistochemistry were 'de-waxed', fixed and rehydrated. In brief, two 5 minute incubations in the citrus-based clearing agent Histolene (Merck, Victoria). The tissue was fixed and re-hydrated with two 5 minute incubations in 100% ethanol, followed by 5 minute incubation in 70% ethanol and 5 minutes in running water. The human foetal tissue sections were incubated with neat GCTM-5 supernatant before addition of an anti-mouse Ig conjugated to Alkaline Phosphatase (AP) (Dako, NSW). The visualisation of the secondary antibody involved detection of AP activity with the SIGMA *FAST:* Fast Red TR/Naphthol AS-MX (Sigma, NSW) substrate and counterstaining with Mayers' Haemotoxylin and 30mM $(NH)_4OH$.

(b) GCTM-5 expression analysis in the seven week human embryo

Preliminary analysis suggests that GCTM-5, an antibody raised against a testicular seminoma, reacts with foetal liver. Further investigation in this study revealed that GCTM-5 is expressed exclusively in the liver of the seven week human embryo, as shown in Figure 4.

GCTM-5 staining was not detected in foetal heart, lung, kidney, CNS and definitive gut, while it appears to be expressed uniformly in the hepatoblasts of the developing human foetal liver. Significantly, sections of human yolk sac failed to show reactivity with GCTM-5.

(c) Liver specific expression of GCTM-5 in the human embryo

Expression analysis in the 7 week human embryo revealed that GCTM-5 is expressed exclusively in the liver. GCTM-5 uniformly stains hepatoblasts of the

developing human liver. Hepatoblasts are bipotential cells of the foetal liver which differentiate to give rise to both hepatocytes and biliary cells. This suggests that GCTM-5 recognises an early liver progenitor population, and indicates GCTM-5 could be a useful tool for the further study of early human embryogenesis; in particular liver formation.

(d) GCTM-5 as a marker for differentiated cells derived from cultured pluripotent stem cell lines

Previous data identified GCTM-5 expression in very rare populations of cultured human ES cells and EC cell line GCT72.

Staining of live cells with GCTM-5 displayed a typical polygonal pattern of staining. The live staining occurs with cells that are not permeablised, through antibody binding to cell surface or pericellular epitopes. The positive GCTM-5 staining of live cells demonstrates that GCTM-5 is a cell surface marker. This property gives rise to the potential use of this marker to isolate populations of cells by immunomagnetic separation, or fluorescence assisted cell sorting (FACS).

Example 4: Diseased Liver Cell Isolation, Culture and Characterisation.

(a) Liver Tissue:

Liver tissue was obtained from the adult and paediatric liver transplant programs at the University & Birmingham Children's Hospitals, NHS Trust Birmingham, UK. Hepatectomy specimens were obtained from Primary Biliary Cirrhosis (PBC, n=6); Alcoholic Liver Cirrhosis (ALD, n=6) and Extra-hepatic biliary atresia (EHBA, n=3). Donor tissue was obtained from the paediatric transplant program when in excess to surgical requirements and served as normal controls (n=5). For immunohistochemistry, tissue was snap frozen and stored at –70°C. For cell isolation, tissue was stored in Dulbecco's Modified Eagles Medium (DMEM, Gibco) at 4°C and used within 48h post-hepatectomy.

(b) Diseased Liver Cell Isolation and Culture

Isolation of HEA- and GCTM-5-positive cells was based on methodology previously described. Briefly, following percoll density gradient centrifugation at 800g for 30 min, the non-parenchymal fraction at the percoll band interface and the 1.04 g ml^{-1} layer was removed. The suspension was divided into 2 equal fractions and cells were further purified using immuno-magnetic separation with either the biliary cell marker, Human Epithelial Antigen-125 (HEA-125, 1:10, IgG1, TCS Biologicals Ltd) or GCTM-5 antibody. The antibody-coated cells were then selected using magnetic dynabeads (subclass IgG, Dynal, Wirral, UK).

Isolated cells from each of the fractions were resuspended in biliary plating media and plated in 12 x $2cm^3$ wells (24 well plates) and incubated at 37^0C with 5% CO_2. After 24-72 h, media was removed and replaced with biliary cell growth media and re-fed on alternate days.

(c) Phenotypic characterisation of cultured cells

After 6 days, cultured cells were stained for expression of specific proteins. The cells were fixed with 70%v/v ethanol and washed twice with phosphate buffered saline (pH 7.4). Primary antibodies CK-19, (IgG1, 1:100, DAKO, High Wycombe, UK), HEA-125 (IgG1, 1:100) both specific for biliary epithelial cells in liver; CK-18 (IgG1, 1:10, DAKO) recognising both hepatocytes and biliary epithelial cells; CD31 (1:100, Dako) a marker for endothelial cells; and GCTM-5 (IgG1, neat) were incubated for 1 h at 25°C. Staining was visualised using the immunoperoxidase Vector Stain ABC Elite kit (Vector Labs, Peterborough, UK).

(d) GCTM-5 expression in normal and diseased liver

In normal liver (age range 5-34y), GCTM-5 was present on the luminal surface of bile ducts and co-localisation was present with the biliary cell marker, CK-19 (Fig 5A). In diseased liver (PBC, ALD), where some intact bile ducts remained, coiocalisation with CK-19 and GCTM-5 was again apparent on bile ducts (Fig 5B, PBC). The ductular reactive cells in all diseased tissue examined (PBC, ALD, EHBA) was similar. GCTM-5 was co-expressed with CK-19 on the majority of the

ductular reactive cells (Fig 5C-E), although some CK-19 positive ductular reactive cells were present closest to the hepatocyte regions.

(e) GCTM-5 positive cells isolated from diseased liver - Phenotype of GCTM-5 cultured cells from cirrhotic liver

The phenotype of cells isolated using GCTM-5 by immunomagnetic separation and grown in culture for 6 days was compared to biliary epithelial cells isolated using HEA-125 from 4 diseased livers (3 PBC and 1 ALD). With time in culture in biliary growth medium, colonies expanded in both groups and gave rise to cultures with remarkably similar properties. When immunostained using the biliary cell markers CK-19 or HEA-125, colonies derived from the isolated GCTM-5 and HEA-125 cells were positive (Fig 6A-D). All cultured cells stained with CK-18 (data not shown). Both groups of cultured cells stained positive for the GCTM-5 antibody (Fig 6E,F) but were negative for the endothelial cell marker CD31 (Fig 6G,H) and a no primary control (data not shown).

(f) GCTM-5 expression in diseased liver

The staining of GCTM-5 in normal and diseased adult liver revealed a pattern of expression which correlates with the previous data showing liver specificity of the marker. The expression of GCTM-5, and its localisation with cytokeratin 19 shows a marker for biliary epithelial cells and ductular reactions in adult liver. GCTM-5 expression was analysed in normal paediatric and adult liver, as well as liver tissue of patients with primary biliary cirrhosis (PBC), extrahepatic biliary atresia (EHBA) or alcoholic liver disease (ALD).

GCTM-5 staining of diseased liver of patients with PBC, EHBA and ALD revealed a localisation to the ductal regions of these tissues. Staining in normal paediatric and adult liver showed a much reduced expression of GCTM-5, in comparison with diseased tissues. This strongly implies that GCTM-5 is recognising an epitope expressed on proliferating liver progenitor cells found predominantly in diseased, regenerating tissue. It is possible that these progenitors are a multipotent

population with the ability to differentiate to biliary cells and hepatocyte, upon which GCTM-5 expression is down-regulated.

In addition, the number of cells expressing the GCTM-5 antigen in the liver is increased in various forms of liver disease. Therefore, the GCTM-5 antibody or derivative reagents may find application in diagnostic histopathology of hepatic or other disorders. Furthermore, it is possible that the GCTM-5 antigen might be shed or secreted from the cell surface. Therefore, the levels of the antigen in blood or other body fluids, which could be measured by radioimmunoassay, ELISA or other assays based on GCTM-5 or derivative reagents, could be used to diagnose or to monitor progression and treatment of hepatic and other disorders.

The GCTM-5 antibody is not ideally a marker of cells of hepatocytic origin in the adult liver given its lack of expression in mature hepatocytes and down-regulation of expression on the hepatocyte-duct interface. GCTM-5 is a marker of a bipotential precursor population originating from the inner ductal region, whose expression is down regulated upon maturation to hepatocyte, but not biliary epithelium.

Staining of GCTM-5 positive isolated cells from PBC diseased liver revealed a remarkably similar expression pattern to HEA-125 isolated cells. GCTM-5+ cells expressed mature biliary markers, CK19 and EMA (epithelial membrane antigen) (data not shown), and HEA-125, but did not express CD31, an endothelial marker. This data suggests that GCTM-5 is a marker for maturing biliary epithelial cells.

From the data accumulated in this study, it is feasible to hypothesise that GCTM-5 is an early endoderm marker, which appears to have liver specificity in the early human embryo. GCTM-5 may, in the future be utilized to isolate such endoderm progenitors from differentiating human ES cells, or disease adult liver tissue.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the

scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the invention.

DATED: 9 October, 2003
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FIGURE 1

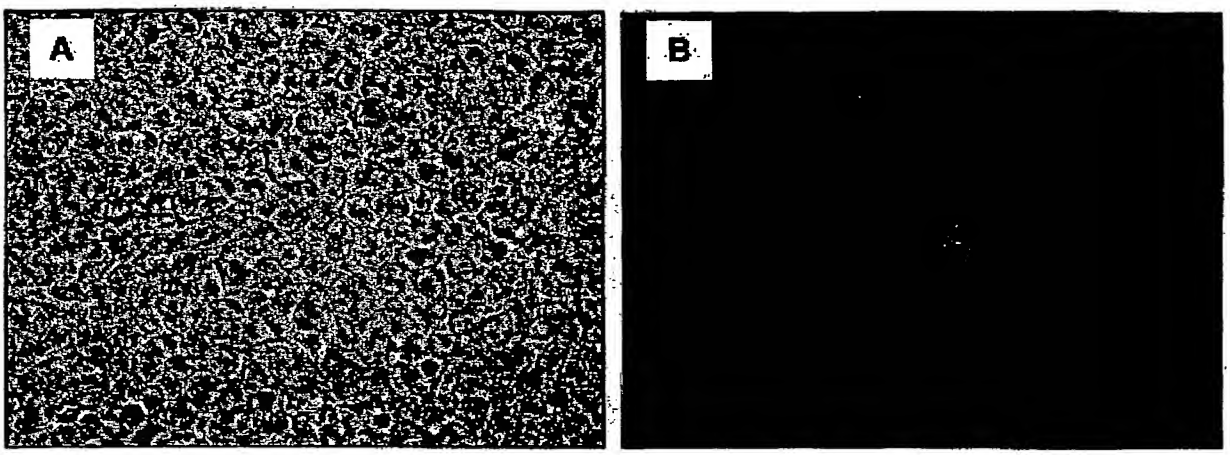


FIGURE 2

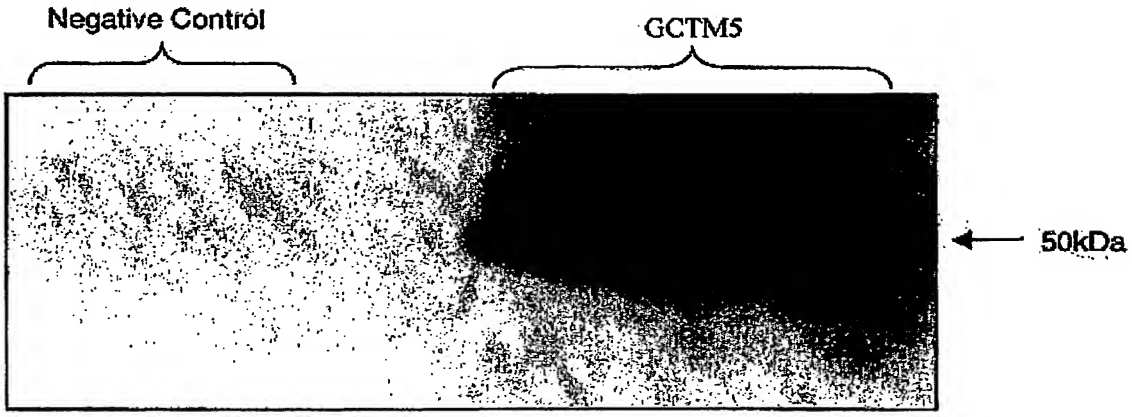


FIGURE 3

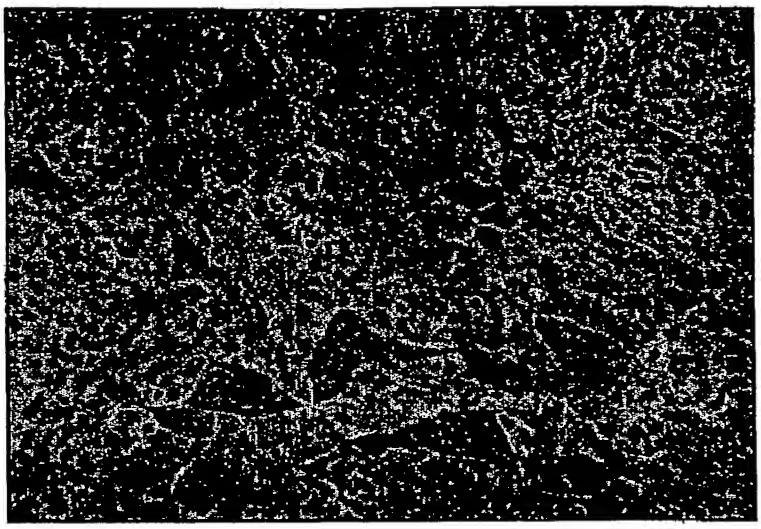


FIGURE 4

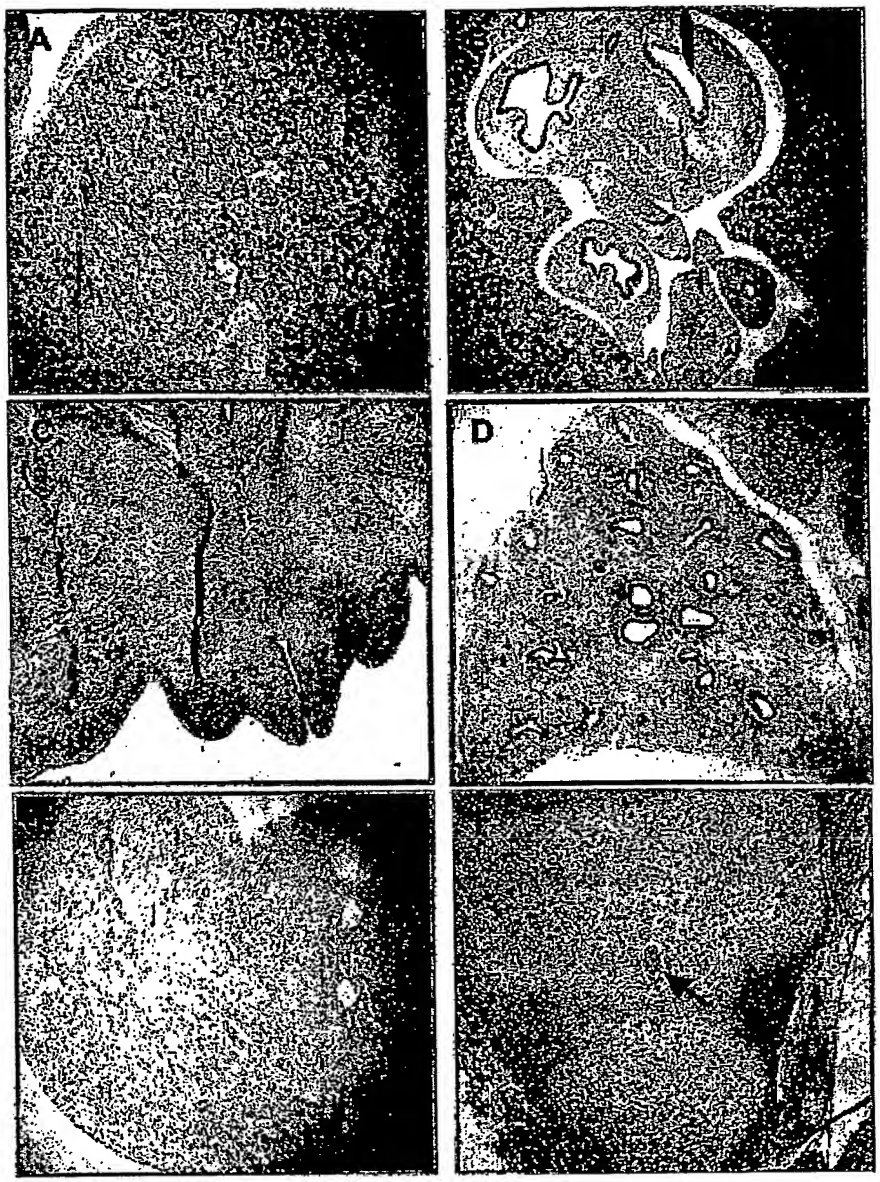


FIGURE 5

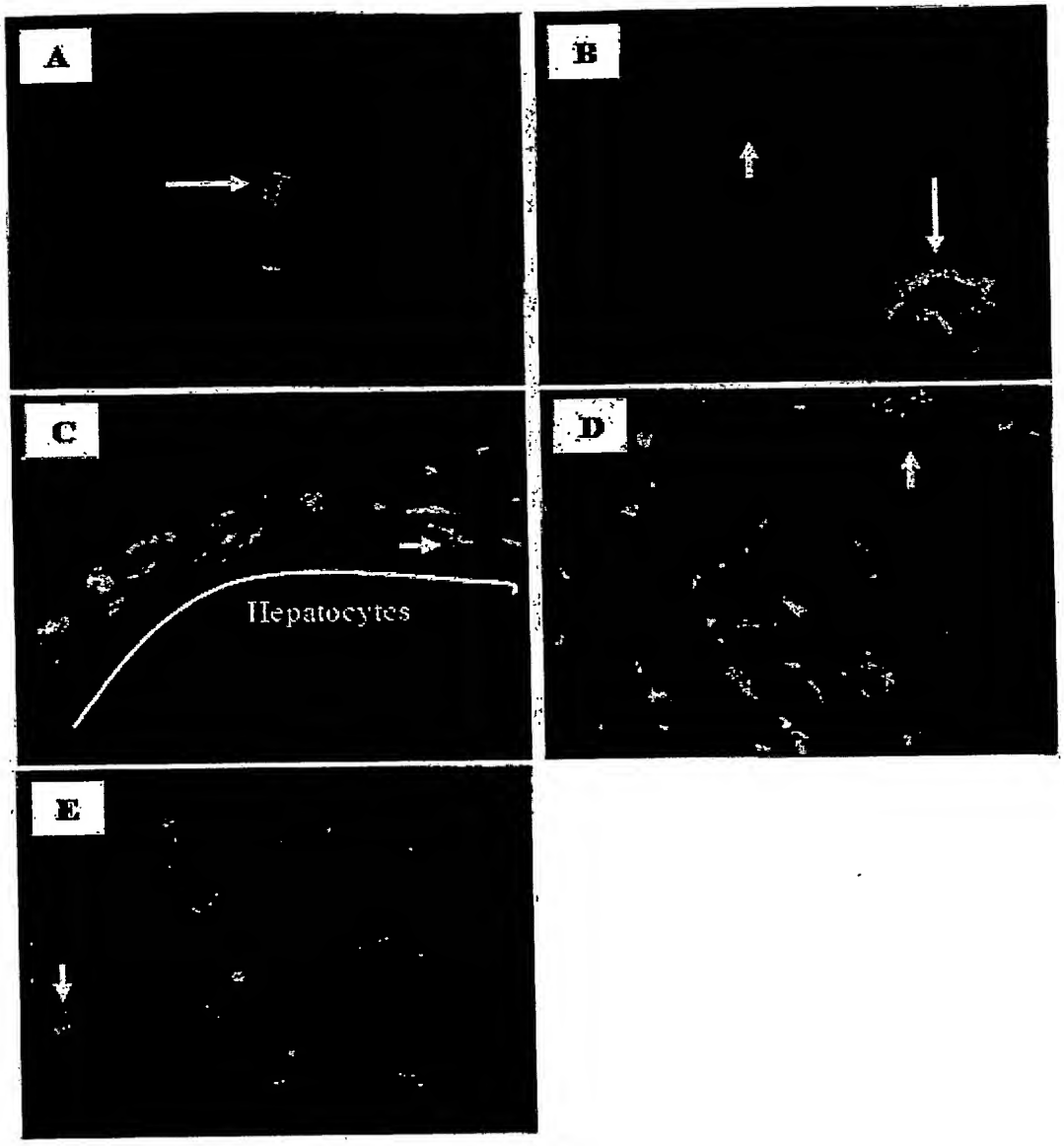
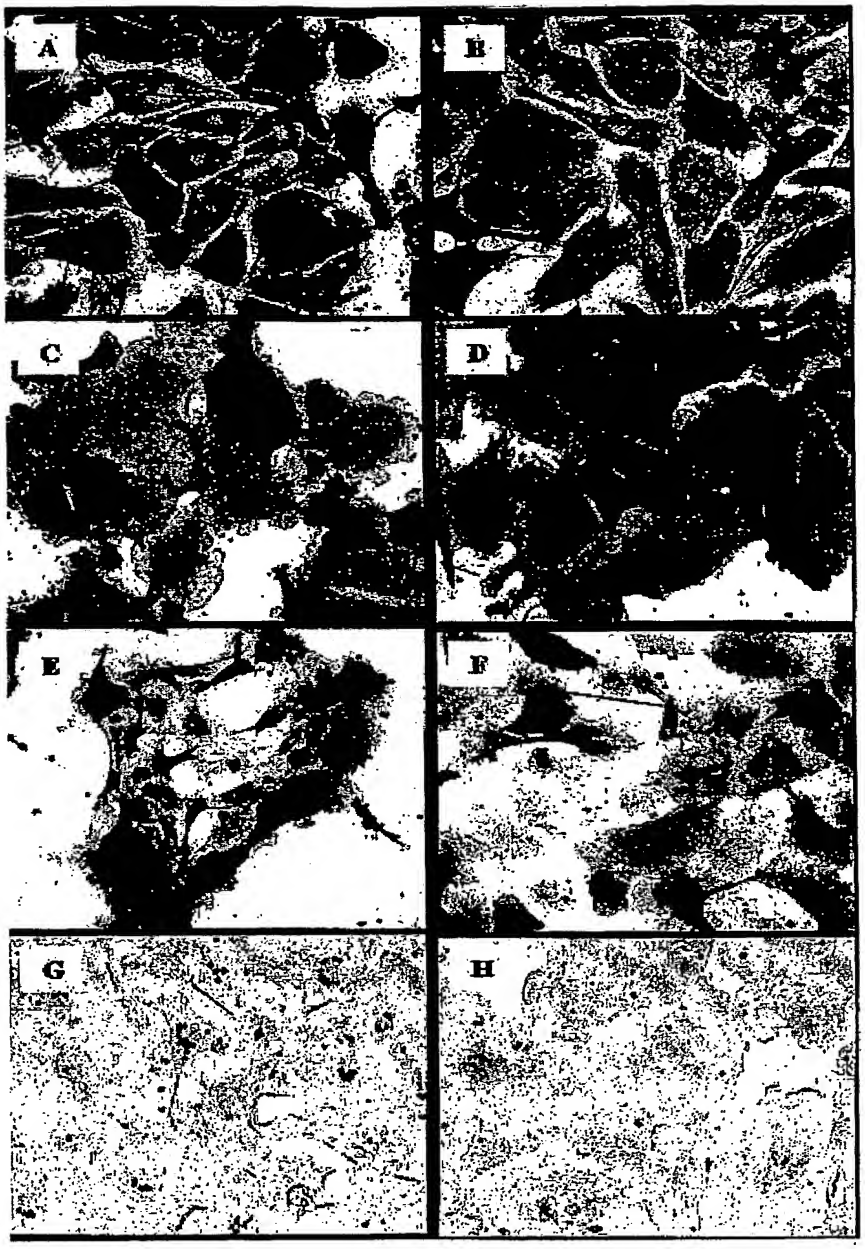


FIGURE 6



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